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ORIGINAL ARTICLE

Molecular characterization of Lolium latent virus, proposed type member of a new genus in the family *Flexiviridae*

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Abstract Lolium latent virus (LoLV) was recently detected in the USA for the first time in ryegrass hybrids (*Lolium perenne* × *Lolium multiflorum*). The genome of one USA isolate, LoLV-US1, has now been fully sequenced. The positive strand genomic RNA is 7674 nucleotides (nt) long and is organized in five open reading frames (ORFs) encoding the replication-associated protein, the movement-associated triple gene block proteins and the coat protein (CP). The genome organization is similar to that of viruses in the genera *Potexvirus* and *Foveavirus*; however, analysis of the complete LoLV genomic sequence, phylogenetic analyses of the deduced amino acid (aa) sequences of the polymerase and the CP, presence of a putative ORF 6, and the in vivo detection of two CPs in equimolar amounts, highlight features peculiar to LoLV. These characteristics indicate that LoLV forms a monotypic group separate from existing genera and unassigned species within the family *Flexiviridae*, for which we propose the genus name Lolavirus. One-step RT-PCR was developed for quick and reliable LoLV detection.

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Introduction

Ryegrasses are used extensively throughout the world because they provide high yields and quality forage/pasture and excellent turf in a wide range of environments. Two species are the most important; *Lolium perenne* L. (perennial or English ryegrass) and *Lolium multiflorum* Lam. (annual or Italian ryegrass) are widely distributed throughout temperate zones on all continents and grown as pastures, amenity grasses, and for soil conservation purposes.

Infection by Lolium latent virus (LoLV), also described as ryegrass latent virus, in *L. perenne* and *L. multiflorum* breeding clones was initially reported in several areas in Europe (Germany, the Netherlands, France, and the UK) [13, 18]. Recently LoLV was reported for the first time in clonally propagated *Lolium* plants from the USA; to date, only partial sequence data has been reported for an isolate of LoLV from the UK that was intercepted in germplasm imported to the USA [19]. Plants infected with LoLV alone exhibited either no symptoms or mild chlorotic flecking that coalesced to form chlorotic to necrotic streaking on the leaves.

Flexiviridae virus isolates are single-stranded RNA viruses able to infect a wide range of plant species. The family includes eight genera, *Potexvirus*, *Mandarivirus*, *Allexivirus*, *Carlavirus*, *Foveavirus*, *Capillovirus*, *Vitivirus*, and *Trichovirus*, as well as the proposed genus *Citrivirus* and several unassigned species. They share genomic organization, strong phylogenetic relationships between replicational and structural proteins and particle morphology [1, 20]. Recently a mycovirus, botrytis virus X, has been shown to have high amino acid (aa) identity to genes from plant "potex-like" viruses [12] and is therefore included here for comparison.

This communication reports partial characterization and the complete nucleotide sequence of LoLV, including study of its genome organization and phylogenetic relationships.

Material and methods

Virus sources and RNA extraction

LoLV isolate US1 was isolated from *L. perenne* × *L. multiflorum* hybrid plant MF-22, one of four hybrids in which the virus was detected by enzyme linked immuno sorbent assay (ELISA) and potexvirus-specific polymerase chain reaction (PCR) [19]. Hybrid MF-22 was the only plant of the four which was not also infected with ryegrass mosaic virus, as determined by ELISA using the potyvirus cross-reactive monoclonal antibody PTY 1 [15] and potyvirus group-specific PCR [6]. LoLV-US1 was maintained in *L. perenne* × *L. multiflorum* and *Nicotiana benthamiana* Domin plants through mechanical inoculation using c. 10 volumes 1% K_2HPO_4 solution with a small amount of celite added as an abrasive. Total RNA extraction was performed using the RNeasy Plant Mini Kit (QIAGEN Inc. Valencia, CA, USA) for cDNA synthesis, or the Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) for a one-step RT-PCR detection assay, following the manufacturer's instructions. Preparations of LoLV particles from infected *N. benthamiana* showing a well-defined chlorotic to white mosaic were obtained, and LoLV RNA extracted as described by Hammond et al. [11].

cDNA synthesis, cloning, and RT-PCR detection

Initial cloning was carried out following cDNA synthesis with an oligo dT primer and M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA), using total RNA from ryegrass MF-22 as template.

Degenerate potexvirus group-specific primers (Agdia Incorporated, Elkhart, IN, USA) were used for PCR with AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) following the suppliers' recommendations, and the PCR products cloned by ligation into pCR2.1 using the TA Cloning Kit (Invitrogen). Additional regions of the genome were cloned using primers derived from the partial sequence of LoLV-UK (DQ333886; and Maroon-Lango, Li, and Hammond, unpublished). The sequences obtained were used to design specific primers able to amplify nine overlapping regions encompassing the entire genome; cDNA was synthesized from RNA extracted from purified preparations of LoLV, using specific primers and M-MLV reverse transcriptase (Promega) following the manufacturer's instructions. One microliter of cDNA was then used as template in a 25 µl PCR with AmpliTaq Gold polymerase (Applied Biosystems). Annealing temperatures and extension times were chosen according to the primer pairs and the expected sizes of the amplified fragments; a touchdown PCR procedure was used to obtain some products. PCR amplified fragments were purified either using QIAquick PCR purification or QIAquick gel extraction kits (Qiagen) and individually cloned into pCR 2.1 vector using the TA Cloning Kit (Invitrogen). Plasmid DNAs from selected clones were amplified using the Big Dye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems) and sequences determined using an ABI Prism 310 (Applied Biosystems). At least four independent clones were sequenced for each region. The 5′ terminal 2088 nt and the 3′ terminal 364 nt of the genome were cloned using the FirstChoice RLM-RACE Kit (Ambion Inc., Austin, TX, USA) using LoLV-specific primers and total RNA from LoLV-infected *N. benthamiana* as template, following the manufacturer's instructions.

One step reverse transcription (RT)-PCR detection of LoLV-US1 was performed using the SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen), following the manufacturer's instructions, with forward primer 5′-TTTGCTGTCGG CCTAGGGATC-3′, reverse primer 5′-CGTTCATAGC TTGCCTCTGTG-3′, and a 60°C annealing temperature. The amplified 806 bp fragment encompasses the TGB2, TGB3 and N-terminal coat protein (CP) regions of LoLV-US1. Extracts of healthy plants and plants infected with LoLV-UK, Alternanthera mosaic virus (AltMV, potexvirus), potato virus X (PVX, potexvirus), phlox virus M (PhlVM, carlavirus), or phlox virus S (PhlVS, carlavirus) were included as controls.

Antiserum production and protein analysis

A preparation of LoLV-US1 particles at c.21.7 mg/ml, was sent to Cocalico Biologicals, Inc. (Reamstown, PA, USA) for use as immunogen following Cocalico's standard protocol for polyclonal rabbit antisera. Test bleeds at 5 and 8 weeks following initial immunization were shown to have significant virus-specific reaction by indirect antigen-coated plate ELISA [15], and the final serum was collected at approximately 3 months. Indirect ELISA was also utilized for LoLV detection, with extracts of healthy plants and plants infected with AltMV, PVX, PhlVM, and PhlVS included as controls.

Total protein extraction was performed using TCA protein precipitation [27] from one leaf-disk of about 35 mg; alternatively a leaf-disk was directly extracted using 80 µl of Smash buffer [9]. For 12% SDS-PAGE total protein samples were boiled for 10′ in 80 µl of Smash buffer, centrifuged briefly and the supernatant (5 µl) used

for gel loading. SDS-PAGE gels were stained (SimplyBlue Safe Stain; Invitrogen) according to the manufacturer's protocol. Western blotting on PVDF membrane (Immobilon-P Transfer Membrane; Millipore, Billerica, MA, USA) was performed using standard procedures, with detection by a goat anti rabbit IgG-Alkaline phosphatase conjugate (Kirkegaard & Perry, Gaithersburg, MD, USA) and BCIP/NBT (Sigma-Aldrich, St Louis, MO, USA) substrate for colorimetric reaction. Total protein from a purified preparation of LoLV was first stained with Sypro Ruby Protein Gel Stain (Invitrogen) for total protein after SDS-PAGE; and then stained with an Emerald Q-Pro kit (Invitrogen) and visualized using a UV trans-illuminator according to the manufacturer's protocol, in order to assay for protein glycosylation.

Sequence analysis

Editing of electropherogram files, multiple sequence alignments and merging of sequences were performed using Chromas version 1.45 (Technelysium Pty Ltd) and DNASTAR-Lasergene v6 (DNASTAR Inc., Madison, WI, USA) using default parameters. Open reading frames (ORF) were identified using ORF Finder; BLAST N, BLAST P, and PASC tools were used for nucleotide or amino acid comparisons with corresponding sequences available in the databases; sequence motifs were identified using CDART; all were accessed from the National Center for Biotechnology Information (NCBI) web site. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [24]; viral species for which a complete genome is available at the NCBI database (official, tentative, newly proposed, and unassigned species within the *Flexiviridae*) were included in the study and were considered according to the VIII ICTV report [1]. Some *Trichovirus*, *Vitivirus*, *Carlavirus*, *and Foveavirus* species for which only the CP sequence is available were included in the study, in order to enlarge the number of species representing these genera. Two *Mycovirus* species, infecting the phytopathogenic fungi *Sclerotinia sclerotiorum* (Sclerotinia sclerotiorum debilitation-associated RNA virus, SsDRV) and *Botrytis cinerea* (Botrytis virus X, BVX) whose encoded proteins show similarities to those of potex-like plant viruses were also included. A complete list of the species used, and their abbreviations, is given in the legend to Fig. 1. Amino acid sequences of complete replicases and CPs were aligned using CLUSTALW with default parameters and BLOSUM protein weight matrix. Trees were created by the Neighbor-Joining algorithm, using JTT matrix and pairwise gap deletion (similarly to the study in [20]). Numbers indicate percentage bootstrap support from 1,000 bootstrap replication; branches with less than 50% bootstrap support were collapsed.

The full nucleotide sequence of LoLV genome is available with GenBank accession number EU489641.

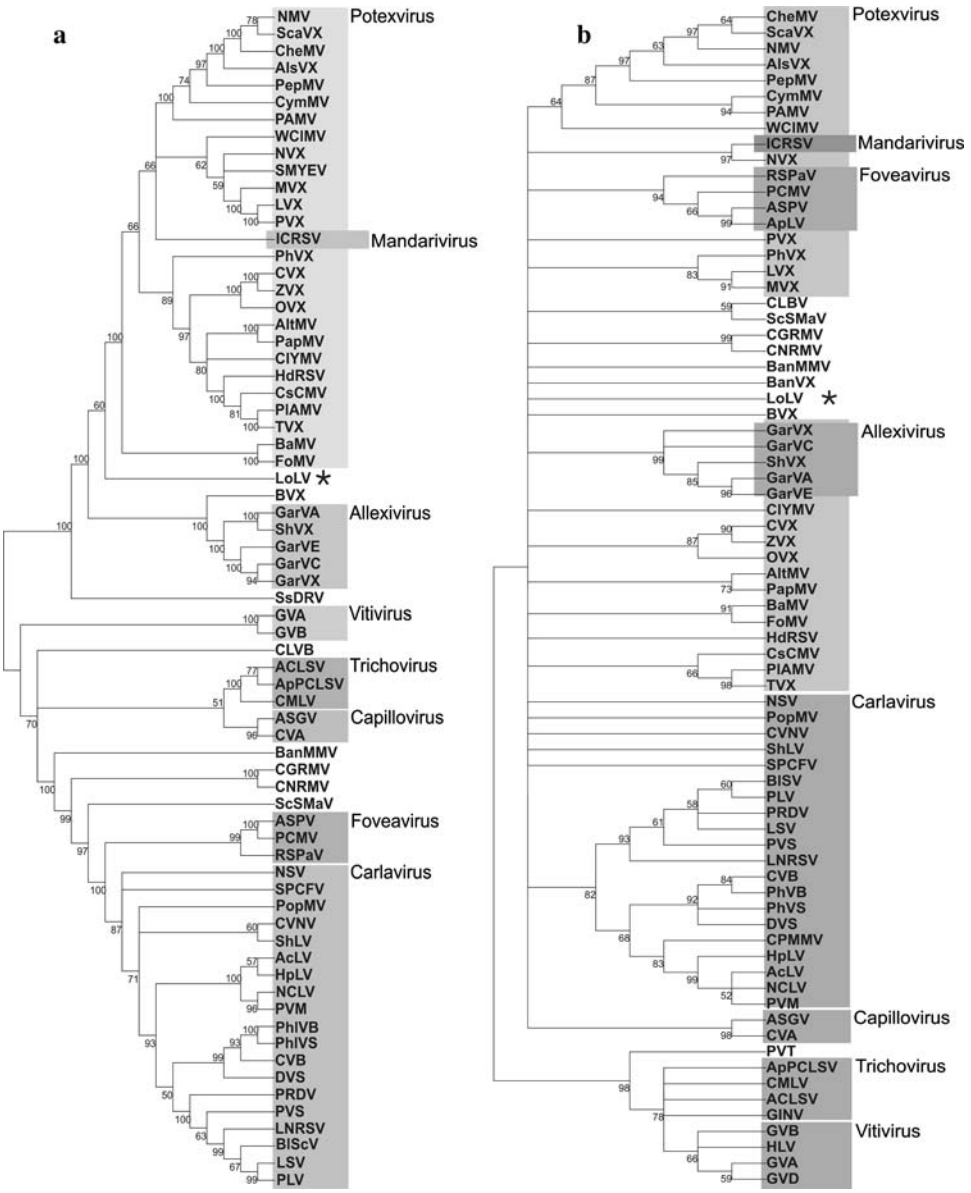
Results and discussion

Antiserum production and Western blotting

A LoLV-US1-specific polyclonal rabbit antiserum raised against purified virus was evaluated by indirect antigen-coated plate ELISA. The pre-immune rabbit serum yielded c.0.05 OD_{405} for a 1:400 dilution against both healthy and infected ryegrass tissue, but c.0.45 OD_{405} against both healthy and infected *N. benthamiana* tissue. However, the antiserum clearly differentiated infected from healthy ryegrass tissue at serum dilutions of at least 1:12,800 (>2.5 OD_{405}, compared to 0.02 for healthy tissue). LoLV-infected *N. benthamiana* was also clearly differentiated from healthy extracts (>2.5 OD_{405} at 1:4,000 dilution compared to <0.05 for healthy tissue) despite the apparent presence in pre-immune serum of antibodies reactive to healthy tissue. For routine ELISA the background reaction was eliminated by dilution of antibody in healthy *N. benthamiana* extracts (1:5 w:v in PBS-Tween); by this means LoLV was readily detected (>2.5 OD_{405} at 1:4,000 serum dilution against LoLV-infected *N. benthamiana* or ryegrass, compared to <0.01 for healthy tissue of either species). A weaker reaction (0.82 OD) was obtained against AltMV-infected *N. benthamiana*, and no reaction to PVX, PhlVS or PhlVM. In reciprocal tests, no reaction to LoLV (<0.01 OD) was detected with antisera to AltMV, PVX, or PhlVS when homologous reactions were each >1.0 OD, and cross-reactions were observed between AltMV and PVX. In tests by immunospecific electron microscopy [13] or ELISA [18], LoLV was not detected by antibodies against any definitive or tentative potexviruses tested; our results also show a lack of relationship to two carlaviruses, but for the first time demonstrates a serological relationship to a definitive potexvirus, AltMV. However, this reaction was not reciprocal; no reaction to LoLV (<0.01 OD) was obtained when the homologous AltMV reaction was >2.5 OD.

In analysis of purified virus preparations two proteins, with apparent molecular weight of about 28 and 33 kDa, were found in essentially equimolar amounts using both protein stain in SDS-PAGE (Fig. 2a) and by Western blotting using LoLV-specific antiserum. Both bands were also detected on western blots of total protein extracts from infected plant tissues, and were not present in healthy plant tissue preparations (Fig. 2c); both proteins also showed moderately positive glycosylation (Fig. 2b).

Owing to the high reproducibility of the equimolar ratio between the two CP bands, in different samples and



conditions, the smaller CP cannot be considered as a degraded form of the large one, and might be explained by the presence of an alternative in-frame start codon leading to the synthesis of a shorter CP sharing the same C terminus (see below). Equivalent dual CP bands were also observed in western blots of LoLV-UK (R. Li; personal communication). The incorporation of two forms of the CP into virions of LoLV appears to be unique among the *Flexiviridae*.

RT-PCR detection

Quick and reliable detection was achieved by a one step RT-PCR in both symptomless ryegrass and symptomatic *N. benthamiana*. The expected PCR product of 806 bp was obtained with total RNA extracts of LoLV-US1 infected plants of both species, and no non-specific reactions were detected. Neither LoLV-UK [19] nor isolates of PVX and AltMV (potexviruses), and PhlVM, and PhlVS (carlaviruses) were detected with this method (Fig. 2d), demonstrating the specificity of the RT-PCR assay. The sequences of LoLV-US1 and LoLV-UK differ in the region of the reverse primer (Maroon-Lango, Li, and Hammond, unpublished).

Genome organization of LoLV

The consensus sequence of LoLV was obtained from at least four cDNA clones representing each of nine overlapping regions. The complete genome consists of 7674

◀ **Fig. 1** Phylogenetic analysis of viruses in the family *Flexiviridae* using the amino acid sequences of the full viral replicase (**a**), and of the coat protein (**b**). The position of LoLV is marked with an *asterisk*. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [24]. Selected bootstrap percentage values (1,000 replicates) are shown. Abbreviations, complete name, taxonomy, and accession numbers of all viruses are listed below. Protein accession numbers, where the complete genome is not available for the virus, are *underlined*. *AcLV* Aconitum latent virus, NC_002795; *AlsVX* Alstroemeria virus X, NC_007408; *AltMV* Althernanthera mosaic virus, NC_007731; *ACLSV* Apple chlorotic leaf spot virus, NC_001409; *ASGV* Apple stem grooving virus, NC_001749; *ASPV* Apple stem pitting virus, NC_003462; *ApLV* Apricot latent virus, AAC16234; *ApPCLSV* Apricot pseudo-chlorotic leaf spot virus, NC_006946; *BaMV* Bamboo mosaic virus, NC_001642; Banana mild mosaic virus, *BanMMV*, NC_002729; *BanVX*, Banana virus X, AAW50962; *BlSV* Blueberry scorch virus, NC_003499; *BVX* Botrytis virus X, NC_005132; *CVX* Cactus virus X, NC_002815; *CsCMV* Cassava common mosaic virus, NC_001658; *CheMVX* Chenopodium mosaic virus X, NC_008251; *CGRMV* Cherry green ring mottle virus, NC_001946; *CMLV* Cherry mottle leaf virus, NC_002500; *CNRMV* Cherry necrotic rusty mottle virus, NC_002468; *CVA* Cherry virus A, NC_003689; *CVB* Chrysanthemum virus B, NC_009087; *CLBV* Citrus leaf blotch virus, NC_003877; *ClYMV* Clover yellow mosaic virus, NC_001753; *CVNV* Coleus vein necrosis virus, NC_009764; *CPMMV* Cowpea mild mottle virus, ABD98451; *CymMV* Cymbidium mosaic virus, NC_001812; *DVS* Daphne virus S, NC_008020; *FoMV* Foxtail mosaic virus, NC_001483; *GarVA* Garlic virus A, NC_003375; *GarVC* Garlic virus C, NC_003376; *GarVE* Garlic virus E, NC_004012; *GarVX* Garlic virus X, NC_001800; *GINV* Grapevine berry inner necrosis virus, BAB47273; *GVA* Grapevine virus A, NC_003604; *GVB* Grapevine virus B, NC_003602; *GVD* Grapevine virus D, CAA69070; *HLV* Heracleum latent virus, CAA55856; *HpLV* Hop latent virus, NC_002552; *HdRSV* Hydrangea ringspot virus, NC_006943; *ICRSV* Indian citrus ringspot virus, NC_003093; *LNRSV* Ligustrum necrotic ringspot virus, NC_010305; *LSV* Lily symptomless virus, NC_005138; *LVX* Lily virus X, NC_007192; *LoLV* Lolium latent virus, EU489641; *MVX* Mint virus X, NC_006948; *NCLV* Narcissus common latent virus, NC_008266; *NMV* Narcissus mosaic virus, NC_001441; *NSV* Narcissus symptomless virus, NC_008552; *NVX* Nerine virus X, NC_007679; *OVX* Opuntia virus X, NC_006060; *PapMV* Papaya mosaic virus, NC_001748; *PLV* Passiflora latent virus, NC_008292; *PCMV* Peach chlorotic mottle virus, NC_009892; *PepMV* Pepino mosaic virus, NC_004067; *PhVX* Phaius virus X, NC_010295; *PhlVB* Phlox virus B, NC_009991; *PhlVS* Phlox virus S, NC_009383; *PlAMV* Plantago asiatica mosaic virus, NC_003849; *PopMV* Poplar mosaic virus, NC_005343; *PAMV* Potato aucuba mosaic virus, NC_003632; *PRDV* Potato rough dwarf virus, NC_009759; *PVM* Potato virus M, NC_001361; *PVS* Potato virus S, NC_007289; *PVT* Potato virus T, BAA01044; *PVX* Potato virus X, NC_001455; *RSPaV* Rupestris stem pitting-associated virus, NC_001948; *ScaVX* Scallion virus X, NC_003400; *SSDRV* Sclerotinia sclerotiorum debilitation-associated RNA virus, NC_007415; *ShLV* Shallot latent virus, NC_003557; *ShVX* Shallot virus X, NC_003795; *SMYEV* Strawberry mild yellow edge virus, NC_003794; *ScSMaV* Sugarcane striate mosaic associated virus, NC_003870; *SPCFV* Sweet potato chlorotic fleck virus, NC_006550; *TVX* Tulip virus X, NC_004322; *WClMV* White clover mosaic virus, NC_003820; *ZVX* Zygocactus virus X, NC_006059

nucleotides (nt) excluding the polyA tail, and is organized into five recognized ORFs, with a putative sixth ORF lacking a clear homolog.

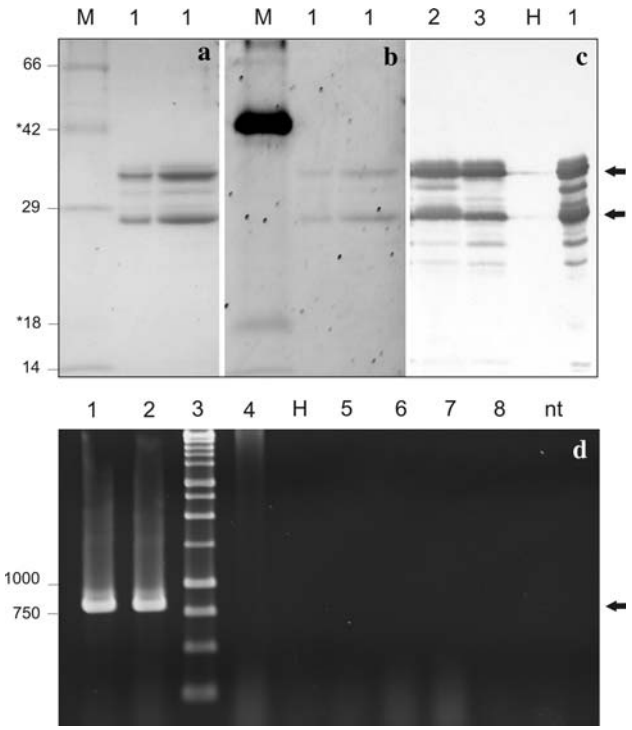


Fig. 2 Protein analysis: **a** Total protein stain and **b** Glycoprotein-specific stain; in *lane 1* total protein extraction at two concentrations from purified LoLV preparation; M, *CandyCane* molecular marker (glycosylated proteins are marked by an *asterisk*). **c** Western blotting; *lane 1*, total protein extract from purified LoLV preparation, *2* total protein extract from LoLV-infected *N. benthamiana* leaves, *3* total protein extract from LoLV-infected ryegrass, *H* total protein extract from healthy *N. benthamiana* leaves as negative control. LoLV antiserum was used at 1:2,000 dilution. *Arrows* indicate the two proteins of about 28 and 33 kDa. RT-PCR: **d** One step RT-PCR detection of LoLV-US1; templates are total RNA extracts from different plants: *lane 1*, LoLV-US1- infected *N. benthamiana*; *lane 2*, LoLV-US1-infected *Lolium* hybrid; *lane 4*, LoLV-UK-infected *L. perenne*; *lane H*, a mixture of uninfected *Nicotiana* and *Lolium* species; *lane 5*, PVX-infected *N. tabacum*; *lane 6*, AltMV-infected *N. benthamiana*; *lane 7*, PhlVS-infected *N. occidentalis*; *lane 8*, PhlVM-infected phlox; *lane nt*, no template, negative control; Invitrogen 1 Kb Ladder in *lane 3* shown in base pairs. *Arrow* indicates the expected 806 bp amplification product

The 5′ untranslated region is 87 nt long starting with the sequence GAAAA, commonly found in potexviruses, thought to be essential for plus-strand RNA synthesis and protein translation [25, 26].

We subjected each of the LoLV ORFs to BLASTP analysis against the databases in order to infer functional and evolutionary relationships; putative functions could be attributed to the corresponding proteins of ORFs 1–5, with limited identity to the equivalent proteins of previously characterized flexiviruses (Table 1).

ORF 1 potentially encodes a 196 kDa protein, corresponding to the LoLV replicase, which is most similar to those of several potexviruses (Table 1); a methyl transferase (MT) domain was identified by visual inspection,

Table 1 Summary of characteristics of LoLV genome ORFs, their predicted protein products, highest amino acid identities to other viral species using BLAST-P search (the three best matches are presented in order of BLAST-P score) and identified domains

ORF/nt coordinates/length	Predicted product	Identities	Domain motifs/aa coordinates [*E* value]
ORF 1 nt 88–5277 5190 nt	196 kDa, replicase	36% ClYMV (*Potexvirus*) 35% ZVX (*Potexvirus*) 42% OVX (*Potexvirus*)	*MT*, Methyl transferase[a], aa 66–234 *AlkB*, (COG3145) Alkylated DNA repair protein, aa 700–831, [2e-6] *Viral helicase 1* (Superfamily 1) (pfam01443) Viral RNA helicase, aa 982–1212, [2e-33] *RNA-dep-RNApol2* (pfam00978), RNA-dependent RNA polymerase, aa 1373–1589, [3e-10]
ORF 2 5349–6155 807 nt	30.5 kDa TGBp1	29% PapMV (*Potexvirus*) 34% GarVB (*Allexivirus*) 32% GarVA (*Allexivirus*)	*Viral helicase 1* (Superfamily 1) (pfam01443) Viral RNA helicase, aa 27–236, [1e-17]
ORF 3 6037–6399 363 nt	13 kDa TGBp2	50% NSV (*Carlavirus*) 52% AcLV (*Carlavirus*) 50% ShLV (*Carlavirus*)	*Plant-vir-prot* (pfam01307) plant viral movement protein, aa 11–108, [2e-19] 2 predicted transmembrane regions[b]
ORF 4 6314–6532 219 nt	7.5 kDa TGBp3	33% BanMMV (*Flexiviridae*) 29% PVM (*Carlavirus*) 32% CVB (*Carlavirus*)	1 predicted transmembrane region[b]
ORF 5 6611–7492 882 nt	31.6 kDa CP	35% BanMMV (*Flexiviridae*) 37% PVX (*Potexvirus*) 32% ShLV (*Carlavirus*)	*Flexi-CP* (pfam00286) viral coat protein from Potex- and Carlavirus, aa 112–248, [3e-27]
ORF 6 7440–7577 138 nt	5.1 kDa NABP		

[a] The methyl transferase domain was identified by visual inspection of the sequence and comparison with the alignment and motifs identified by Rozanov et al. [23]

[b] Prediction of transmembrane regions was performed using the DAS-TMfilter Server [8]

while *AlkB*, *Viral helicase I* and *RNA-dep-RNApol2* domains were identified using CDART (Table 1). The various MT motifs were more similar to the "tymovirus" than the "alphavirus" consensus sequences identified by Rozanov et al. [23], as expected for the *Flexiviridae*. AlkB domains are found in only a subset of the *Closteroviridae* (*Ampelovirus*), and particularly in the *Flexiviridae* genera *Carlavirus*, *Foveavirus*, *Mandarivirus* (monotypic), *Trichovirus*, *Vitivirus*, with limited occurrence in *Allexivirus* (1 of 5 genomes examined) and *Potexvirus* (3 of 17 genomes); *Alk*B homologs are of recent origin within the *Flexiviridae*, and may function in RNA demethylation to counter post-translational gene silencing of viral RNA [3] or pesticide-induced methylation [5]. Viruses with *Alk*B domains infect a range of woody perennial crops in which usage of pesticides that may cause DNA and RNA methylation is common [5]. Ryegrass, though not woody, is also a perennial crop in which multiple pesticide applications are likely over the life of the crop.

ORFs 2, 3, and 4 overlap in different reading frames, and encode the putative triple gene block (TGB) proteins presumed to be involved in virus movement [4, 21, 25]. Each of the TGB proteins show different degrees of identity to the equivalent proteins of various genera of the *Flexiviridae* (Table 1).

ORF 5 encodes a deduced protein of about 31.6 kDa, the putative CP, driven by the "primary" initiation codon (79 nt downstream of ORF 4; in the context UAUAUGUC) identified by the ORF finder program (NCBI). This context, and that of the polymerase, differ from the higher plant mRNA consensus [16]. Interestingly, a second AUG codon, in frame with the first, and in a better context (CCCAUGGC) can be observed 141 nt downstream. As noted above, two forms of the CP were detected in both infected plants and purified virions at equimolar levels. There is some discrepancy between the predicted (31.6 and 26.8 kDa) and apparent (c.33 and c.28 kDa) sizes of the two CP forms, possibly due to post-translational glycosylation (Fig. 2b) at one or more of several potential sites.

The in vivo expression of CP from a non-AUG start codon, together with a truncated form of CP from a

downstream AUG, has recently been observed for Peach chlorotic mottle virus, a member of the genus *Foveavirus*, [14]. The presence of two in-frame putative CP start codons was observed for the potexvirus Plantago asiatica mosaic virus, in which the CP ORF slightly overlaps the TGB3 ORF [17], without evidence of expression of two forms of CP. Foxtail mosaic virus has been reported to have an ORF 5A expressed in vivo, with an initiation codon within the TGB3 gene and in-frame with the ORF 5 CP gene; however, the ORF 5A initiation codon is upstream of the start of the CP subgenomic RNA, and mutants in which translation of the 5A protein was knocked out were still able to infect plants systemically [22]. In none of these cases has incorporation of two forms of CP into virions been demonstrated. The mechanism of translational regulation and functional significance of the equimolar occurrence of the two LoLV CP forms is currently unclear, but appears to be an interesting and unique feature of LoLV.

A sixth small ORF partially overlaps the CP ORF; the predicted 45 aa, 5.1 kDa product is highly basic (pI 9.56) with three Cys, two His, and four Arg residues spaced through the 45 residues (protein ID ACA53379), and has no significant homology to proteins in the database. The putative ORF 6 peptide is of interest because of its position overlapping the C-terminal region of the CP and the 3′ UTR; the very basic character suggests that it may act as a nucleic acid binding protein (NABP). The spacing of His and Cys residues differs from those observed for presumptive NABPs of other flexiviruses [7, 10, 20] and the predicted 5.1 kDa peptide of LoLV is much smaller than the c.10–23 kDa NABPs of other flexuous viruses. However, the genome location and putative NABP function suggest that the LoLV ORF 6 peptide, if expressed, may serve as a suppressor of RNA silencing.

Several other small ORFs (38–61 aas) were observed in alternate reading frames within ORF 1 and ORF 5, but no significant homologies or characteristics of these ORFs could be identified.

The LoLV 3′ untranslated region is 97 nt (if ORF 6 is considered) or 182 nt long (if ORF 6 is not included) and is followed by a poly A tail.

Phylogenetic analysis

The flexuous filamentous particles [18] and genome organization of LoLV are characteristic of the family *Flexiviridae* [2]. We constructed phylogenetic trees to examine the relationship of LoLV to other characterized viruses. Phylogenetic trees were obtained by aa sequence comparison of 69 replicases (Fig. 1a) and 74 CPs (Fig. 1b) of different species and genera in the *Flexiviridae* and of two mycoviruses (the mycovirus SsDRV lacks an identified CP).

In the phylogenetic tree based on replicases (Fig. 1a), assigned species grouped according to genus affiliation with high percentages of bootstrap replication supporting branch nodes. The LoLV branch is positioned within the cluster of potexvirus-like viruses [20] in accord with the highest aa identities (Table 1; Fig. 1a), but is distinct from the *Potexvirus* and *Allexivirus* clades, the monophyletic *Mandarivirus* branch, and the mycovirus BVX, which clusters with the *Allexivirus* clade. The other mycovirus, SsDRV, is also associated with the potexvirus-like cluster, but separated from the genera with high bootstrap support. The LoLV replicase was clearly separated and far distant from those of the other unassigned flexivirus species *Banana mild mosaic virus* (BanMMV), *Citrus leaf blotch virus* (CLBV, proposed genus *Citrivirus*), *Sugarcane striate mosaic associated virus* (ScSMaV), *Cherry green ring mottle virus* (CGRMV), and *Cherry necrotic rusty mottle virus* (CNRMV), which clustered with the *Carla-*, *Fovea-*, *Capillo-*, *Tricho-*, and *Vitivirus* genera (Fig. 1a).

In the CP phylogenetic tree (Fig. 1b), the LoLV branch is positioned among the cluster comprising all genera except *Vitivirus* and *Trichovirus*, together with other unassigned viruses such as BanMMV, Banana virus X (BanVX) and the mycovirus BVX.

The whole genome comparison tool PASC has previously been successfully applied to geminiviruses, potyviruses, flexiviruses, and papillomaviruses. The complete genome sequence of LoLV was analyzed through pairwise global alignment on all complete genomes (121) available for the family *Flexiviridae* using PASC. The best match of LoLV to the genomes within the *Flexiviridae* was to *Indian citrus ringspot virus* (genus *Mandarivirus*, showing 55.24% identity), followed by six potexviruses and a vitivirus. Out of 36 full genome pairwise comparisons with identities between 55% and 55.5%, 23 pairs (about 64%) were between isolates of different genera or unassigned species, while only 13 pairs were between species within a genus. The low best identity value found according to PASC tool suggests that LoLV most likely belongs to a new genus within the family *Flexiviridae*.

Species in different genera of the *Flexiviridae* typically share less than 40% aa identity, in both the CP and replicase proteins [20]. The highest identity score for LoLV CP aa sequence was 35% to the unassigned BanMMV, below the level typical of viruses within any of the established flexivirus genera. The highest identity score for LoLV replicase was 36% with ClYMV (*Potexvirus*), even though as a third score 42% identity was obtained with OVX (*Potexvirus*), a value slightly over the threshold. It is of interest that ClYMV is among those few potexviruses with an AlkB domain in the replicase. However, the low level of

identity in both CP and replicase strongly suggests that LoLV is not a member of any described genus.

The genome organization of LoLV is typical of the *Flexiviridae*. Phylogenetic analysis using replicase, the principal determinant in the evolutionary framework of positive-strand RNA viruses, and CP clearly distinguish LoLV from both established genera and unassigned members of the *Flexiviridae*. The presence of two forms of CP that are incorporated into virions is a feature exclusive to LoLV within the *Flexiviridae*, the function of which is still unknown.

We therefore propose that LoLV represents a new, monotypic genus in the family *Flexiviridae*, for which we propose the name Lolavirus, derived from Lolium latent virus.

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